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**14. ABSTRACT:** Breast cancer often occurs when the proteins that regulate normal epithelial cell division become dysregulated. This proposal examines the role of the cell cycle regulatory proteins, human cyclin-dependent kinase subunits (Cks1 and Cks2) in human breast cancer. The overexpression of Cks genes in breast tumor tissue and the role of Skp2 in tumorigenesis, suggests that Cks and Skp2 levels must be strictly regulated for proper cell cycling. We hypothesize that aberrant Cks protein expression and function contributes to breast carcinogenesis, at least in part, by its ability to interact with Skp2. In year one of this project, we have determined the levels of Cks mRNA and protein in (i) breast cancer cell lines and (ii) normal versus tumor breast tissue. We have also developed breast cancer cell lines that overexpress cks1, cks2, or skp2, and cells that co-overexpress cks1 and skp2. In the coming year, we will focus on characterizing the breast cancer cell lines developed that stably overexpress the aforementioned cell cycle proteins, using both in vitro and in vivo techniques.

**15. SUBJECT TERMS**
Tumor Pathobiology, Breast Cancer, Cell Cycle, Molecular Biology, Protein Structure, Protein, Protein Interactions, Biophysics, Go-Model, Normal Mode Analysis

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## INTRODUCTION:

Cks1 and Cks2 are evolutionarily conserved proteins that interact physically with Cdks and are critical for timely progression through the cell cycle (1). Our research focuses on the role of Cks1 in $SCF^{Skp2}$-mediated degradation. p27, a substrate of $SCF^{Skp2}$, is frequently expressed at low levels in breast tumors and this phenotype is associated with tumor progression and poor patient outcome (*1*). Although the molecular mechanism behind p27 alterations is unknown, one might speculate that it could be due to increased proteolysis as a result of elevated expression of Skp2 and/or Cks1. Indeed, Skp2 is overexpressed in primary breast tumors (*2*) and this phenotype has been shown experimentally to be oncogenic (*3, 4*). The essential role of Cks1 as an adaptor in $SCF^{Skp2}$-mediated ubiquitination of p27 suggests that it may cooperate with Skp2 in breast cell malignancy. It is clear that a more comprehensive analysis of the role of Cks proteins in human breast tumors is warranted, since knowledge gained from this research could lead to a better understanding of the mechanisms underlying breast tumor progression and yield putative diagnostic markers and/or therapeutic targets for treating the disease. In fact, several anti-cancer drugs such as transforming growth factor-beta, vitamin D analogs, and compounds that activate the mitotic spindle checkpoint, all reportedly decrease Cks levels (6, 7).

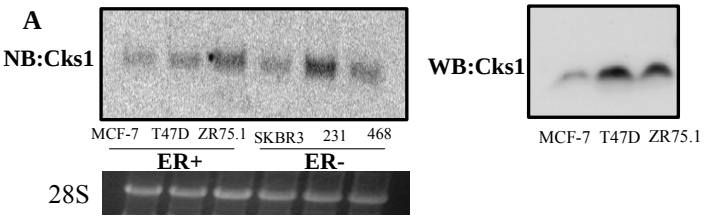
## BODY:

***SOW: Examination of the Cks1-Skp2 regulatory pathway in human breast cancers.* (Months 1-9).**

1- Expression of Cks1 and Cks2 *in vitro* and *in vivo*

We have examined Cks expression by Northern blot and Western blot analysis, in various breast cancer cell lines known to be estrogen receptor (ERα)-positive (MCF-7, ZR75-1, T47-D) and ER-negative (SKBR3, MDA-MB-231, MDA-MB-468). There does not appear to be a tight correlation between Cks1 protein level and ER status (Figure 1a). However, the training that I received in breast clinicopathology (see below) enabled me to determine a positive correlation between Cks2 and ERα status in primary breast tumor samples (Figure 1b). Furthermore, *in vitro* data further suggests that Cks2 is an estrogen-responsive gene (Figure 1c).

**A**




**B**

Association between Cks2 expression and Estrogen Receptor status

| | | NTILES of CKS2 | | Gesamt |
|---|---|---|---|---|
| | | 1 | 2 | |
| ER_STAT | 0 | 17 | 7 | 24 |
| | 1 | 6 | 19 | 25 |
| Gesamt | | 23 | 26 | 49 |

p = 0.002 (Fisher's Exact)

| | | Regressions koeffizientB | Standardf ehler | Wald | df | Sig. | Exp(B) | 95.0% Konfidenzintervall für EXP(B) Unterer Wert | Oberer Wert |
|---|---|---|---|---|---|---|---|---|---|
| Schritt 1 | ER_STAT | 1.910 | .925 | 4.262 | 1 | .039 | 6.752 | 1.101 | 41.396 |
| | PR_STAT | -.056 | .895 | .004 | 1 | .950 | .946 | .164 | 5.461 |
| | NAGEATDI | .494 | .685 | .521 | 1 | .471 | 1.639 | .428 | 6.269 |
| | Konstante | -1.527 | 1.035 | 2.174 | 1 | .140 | .217 | | |

**C**

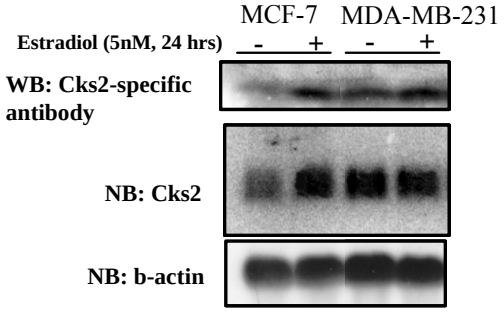



**FIGURE 1: Expression of Cks1 and Cks2 in primary breast cancers and cell lines**

A. Cks1 expression in breast tumor cell lines. Northern blot (NB) was used to determine Cks1 mRNA levels (*left panel*). Western blot (WB) was used to assess Cks1 protein levels (*right panel*). 28S was used as a loading control for NB.

B. Statistical analysis of Cks2 in breast cancer. Logistic regression analysis shows that Cks2 expression correlates with ER status (adjusted for progesterone receptor (PR), age at diagnosis (low/high)).

C. Estrogen stimulates Csk2 expression in breast tumor cells. MCF-7 or MDA-MB-231 cells were treated with estradiol and the expression of Cks2 was assessed by WB (*top panel*) and NB (*lower panel*).

2- Analysis of human breast tissues

In keeping with the proposed aim to investigate the Cks1-Skp2 regulatory pathway in breast cancer, we examined the expression of Cks1, Skp2, and p27 in primary human breast tumor samples obtained from the SKCC tumor bank. Cks1 and Skp2 proteins were found to be overexpressed in breast tumor samples, but no significant correlation was found with p27 level (Figure 2).

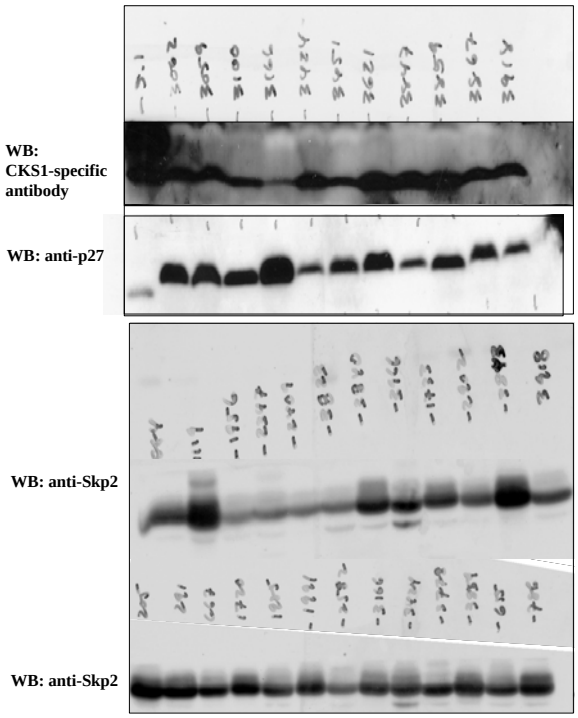



**FIGURE 2: Expression of Cks1, Skp2, and p27 in breast tumor samples available from the SKCC tumor bank.**
The protein level of cks1 was assessed by WB using an antibody which was developed in our lab and is specific for cks1 (top panel). The protein level of p27 (middle panel) and of skp2 (lower panel) were also examined by western blot.

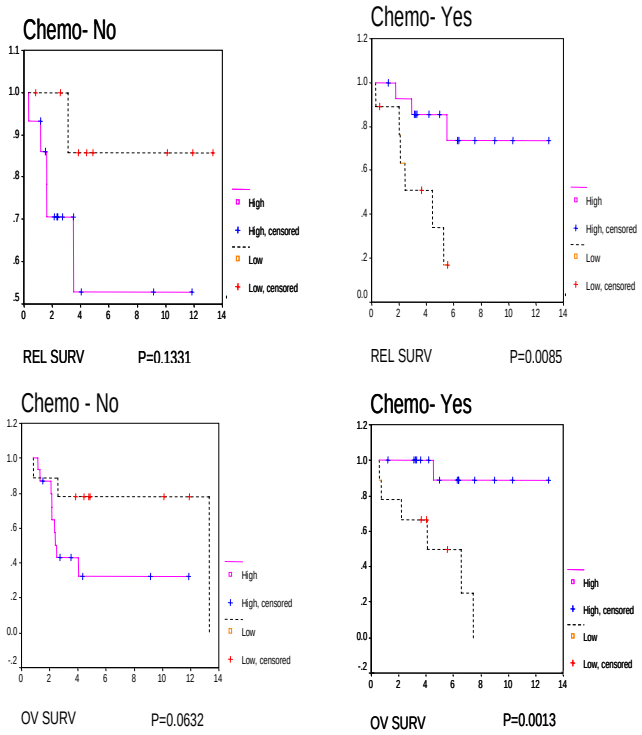



**FIGURE 3: Kaplan-Maier plots for Cks1 in patients with/without adjuvant chemotherapy.**
Overall survival (ov surv) and disease-free survival (rel surv) are significantly associated with Cks1 expression.

In performing the experiments in Aim 1, I received training in breast clinicopathology from the SKCC tumor bank director to pathologically evaluate breast tumors and correlate protein expression levels with tumor pathobiology, prognostics, and other clinical correlates. This training has enabled me to characterize breast tissue samples, by grading tumors using standard criteria: mitotic index, pleiomorphism, and differentiation. Using this knowledge, we found that elevated Cks1 protein level is significantly associated with tumor differentiation (p= 0.002, Fisher's Exact test) and positive response to adjuvant chemotherapy (p= 0.0013, Cox Regression, Fig. 3).

***SOW: Determine if Cks1 cooperates with Skp2 in breast epithelial cell transformation.* (Months 6-20).**

1- *In vitro* cell-based model to study the Cks-Skp2 regulatory pathway

We have successfully created MCF-7 cell lines that overexpress Cks1 or Cks2 to various degrees (Figures 4a and 4b). We have also constructed stable cell lines that overexpress Skp2 alone or co-overexpress Cks1 and Skp2 (Figure 4c). We are currently characterizing these cell lines in more detail.

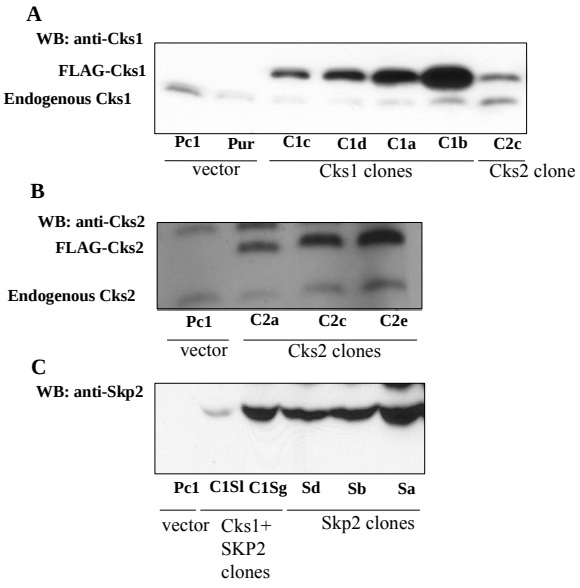



**FIGURE 4: Construction of MCF-7 cells stably overexpressing Cks1, Cks2, or Skp2.**

A. Pc1 and Pur cells are MCF-7 cells stably expressing the empty vector pcDNA3. MCF-7 cells stably overexpressing Cks1 are labeled: C1c, C1d, C1a , and C1b. The WB was probed with a Cks1-specific antibody (developed by us). There is minimal cross reactivity with Cks2 (see C2c clone overexpressing Cks2) .

B. MCF-7 cells stably overexpressing Cks2 are labeled: C2a, C2c, and C2e. The WB was probed with a Cks2-specific antibody (developed by us).

C. MCF-7 cells stably overexpressing Skp2 are labeled: Sd, Sb, and Sa. MCF-7 cells stably overexpressing Cks1 and Skp2 are labeled: C1Sl, and C1Sg.

## KEY RESEARCH ACCOMPLISHMENTS:

1- There is no correlation between Cks1 levels and ER levels in cultured cell lines.
2- Estrogen increases Cks2 mRNA and protein levels in breast cell lines.
3- A strong correlation exists between Cks1 protein expression and disease free survival following adjuvant chemotherapy in patients with breast cancer.
4- Breast cancer cell lines stably overexpressing Cks1, Cks2, Skp2, or Cks1+Skp2 have been made, and are ready to be characterized.

## REPORTABLE OUTCOMES:

Conferences attended / Oral Presentations

1- Invited speaker for the San Diego Black Nurses Association Conference. Title of talk: "Breast Cancer Research: San Diego Overview". February 18, 2006.
2- Keystone Symposium- Ubiquitin and Signaling. February 4 - 9, 2007 (Big Sky, Montana)

## CONCLUSION:

It is important to determine the functional outcome of Cks1 overexpression in breast cancer, since this may provide a novel target for therapeutic intervention. To this end, in the coming year we will focus on characterizing the breast cancer cell lines developed that stably overexpress Cks1, using both *in vitro* and *in vivo* techniques. Furthermore, an important step in research is to bring awareness into the community of the work being done to help eradicate breast cancer, and thus aside from the research outlined in this proposal, I have continued my volunteer work for the Susan G. Komen Breast Cancer Foundation. I will continue to give presentations to the San Diego non-scientific community and health care professionals regarding the potential impact of my research findings, as well as the research being conducted from other labs in the San Diego area.

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